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Identification of three additional *femAB*-like open reading frames in *Staphylococcus aureus*

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Abstract

Three new proteins, FmhA, FmhB and FmhC, with significant identities to FemA and FemB were identified in the *Staphylococcus aureus* (ATCC 55748) genome database. They were mapped to the *Sma*I-C, *Sma*I-H and *Sma*I-A fragments of the *S. aureus* 8325 chromosome, respectively. Whereas insertional inactivation of *fmhA* and *fmhC* had no effects on growth, antibiotic susceptibility, lysostaphin resistance, or peptidoglycan composition of the strains, *fmhB* could not be inactivated, strongly suggesting that *fmhB* may be an essential gene. As deduced from the functions of FemA and FemB which are involved in the synthesis of the peptidoglycan pentaglycine interpeptide, FmhB may be a candidate for the postulated FemX thought to add the first glycine to the nascent interpeptide.

Keywords: *Staphylococcus aureus*; Peptidoglycan; Methicillin resistance; FemX; Pentaglycine interpeptide

1. Introduction

The acquisition of the additional, low affinity penicillin binding protein PBP2′ (PBP2a) is the prerequisite for methicillin resistance in *Staphylococcus aureus*. In the presence of β-lactams, PBP2′ is thought to substitute for the cell's normal PBP functions. In addition to PBP2′, the level of methicillin resistance (a strain-specific characteristic) also depends on *fem* or *aux* factors which are part of the genome of susceptible and resistant strains. Most of these factors have functions in, or affect indirectly, cell wall metabolism (reviewed in [1]). The highest impact on methicillin resistance is shown by the *femAB* operon, whose inactivation abolishes methicillin resistance but still allows PBP2′ production [2]. FemA and FemB are proteins involved in the formation of the peptidoglycan pentaglycine interpeptide. They presumably catalyze and/or control the incorporation of Gly2-Gly3 (FemA), and Gly4-Gly5 (FemB). A third factor (FemX) responsible for the attachment of the first glycine residue to the ε-amino group of lysine of the nascent interpeptide has been postulated

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[3]. Because of similar functions, FemX was speculated to have some sequence similarity with FemA and FemB. Moreover, since *femAB* inactivation also strongly impairs growth, *femX* was thought to have an even greater impact on the cell's viability and to be a lethal target. Low stringency hybridizations indicated that here may be sequences with some homology to *femA* and *femB* in *S. aureus* (B. Berger-Bächi, unpublished results). We searched the *S. aureus* genome for *femAB*-like genes and identified a candidate sequence for *femX* that seems to be essential for *S. aureus*.

2. Materials and methods

2.1. Strains, growth conditions and resistance tests

Protein sequence searches were done on a database derived from the genome sequence of a methicillin-resistant (Mc^r) clinical isolate of *S. aureus* (ATCC 55748). Gene disruption experiments we done in Mc^r *S. aureus* BB270, a derivative of NCTC 8325 [2]. Cells were grown in LB broth at 37°C. Hypertonic media were used when mentioned: medium 1 consisted of 3.7% BHI supplemented with 5% NaCl, 5% sucrose, 0.5% yeast extract, and 0.5% BSA [4]; medium 2 consisted of 2% BHI supplemented with 0.5 M sodium succinate. Minimal inhibitory concentrations (MICs) were determined by microbroth dilution [5] or with E-strips (Solna, Sweden, [6]) on LB plates containing the appropriate antibiotic where needed. The MIC of lysostaphin (AMBI, Trowbridge, UK) was determined by microbroth dilution at 35°C in LB broth supplemented with an antibiotic where required.

2.2. Plasmid preparation, transduction and transformation

For DNA manipulations, the recommendation of the enzyme manufacturer and the protocols of Maniatis et al. [7] were followed. The modifications needed for lysing *S. aureus* by lysostaphin have been described earlier [8]. Preparation and separation of chromosomal *Sma*I digests by pulsed field gel electrophoresis was as described by Wada et al. [9]. Recombinant plasmids were first electroporated in restriction-negative, modification-proficient RN4220 and then transferred by phage 80α mediated transductions into *S. aureus* BB270 as described earlier [2].

2.3. Gene disruption

For gene disruption experiments, internal fragments (~650–700 bp) of the *fmh* genes were amplified by PCR and cloned into the multiple cloning site of the *Escherichia coli- S. aureus* shuttle vector pOX7 [10] which carries an erythromycin resistance marker for selection and is temperature-sensitive (ts) for replication in *S. aureus* and ampicillin-resistant in *E. coli*. The following primers were used for amplifying the internal fragments from strain BB270: MT3 (5′-GGGGATCCGTTGATTTAAAAGGCGATGTG-C-3′) and MT4 (5′-GCCTGCAGAATTTGTTCTA-TTGTATTGTCG-3′) for *fmhA*, MT15 (5′-GC-CTGCAGCAGAAACAAAGAAATTAACTGG-3′) and MT16 (5′-GCCTGCAGATTTAGCTATTTC-GGCATGAAG-3′) for *fmhB*, and MT5 (5′-CCG-GATCCCAGTGATAGTAAAGATGCTAGG-3′) and MT6 (5′-GCCTGCAGATCCGCCTCATTAC-TAAAGACG-3′) for *fmhC*. After cloning in *Escherichia coli* the plasmids were electroporated into restriction-negative *S. aureus* RN4220 and subsequently transduced into the Mc^r strain *S. aureus* BB270. To promote chromosomal integration of the plasmid, strains were grown in LB containing 20 μg erythromycin ml^{-1} at 30°C to stationary phase. The culture was diluted 1:200 into fresh medium, incubated at 30°C for 3 h, and appropriate dilutions were spread on LB agar plates containing 10 μg erythromycin ml^{-1} and incubated at 42°C. Furthermore, we tried to inactivate *fmhB* using a double crossover strategy by replacing an internal ~350-bp fragment of *fmhB* by an *ermB* cassette. The N-terminal part spanning the promoter and ribosome binding site and the C-terminal part including the stop codon and beyond of *fmhB* were amplified with MT19 (5′-GCGAATTCACACGA-AACATAGATATAAAATG-3′)/MT20 (5′-GCGG-TACCCCCAGTGATTTTCATTAATTC-3′), and MT21 (5′-CCGGATCCGCAAAATATGATTAAT-GATGC-3′)/MT22 (5′-CGCTCTAGACCCAAAG-AAAATTGTAATAGC-3′), respectively, yielding approximately 600-nt fragments that were cloned to

the left and right of the *ermB* cassette into the ts *E. coli-S. aureus* shuttle vector pBT2 [11] which is chloramphenicol-resistant in *S. aureus*. Selection for integration of the *ermB* cassette by double crossovers in the chromosome of strain BB270 was by sequential dilution and growth in the presence of 2.5 μg ml^{-1} erythromycin at the nonpermissive temperature of 42°C followed by one growth cycle in the absence of erythromycin and a final plating on erythromycin plates as described by Brückner [11]. The colonies were tested for loss of the plasmid by replica plating on LB agar plates containing 10 μg chloramphenicol ml^{-1} and 2.5 μg erythromycin ml^{-1}. A double crossover event should yield chloramphenicol-sensitive and erythromycin-resistant colonies. As alternative selection media, hypertonic media 1 and 2 were used to stabilize the putative deleterious mutants; or lysostaphin, a glycylglycine endopeptidase, was added at a concentration of 8 μg ml^{-1} to the growth medium to promote and select mutants with altered or shortened pentaglycine interpeptides, where indicated.

2.4. Cell wall composition

The peptidoglycan was isolated and digested with muramidase into muropeptides as described [2]. Reduced muropeptides were separated by reverse phase HPLC [12]. Strains carrying inactivated *fmhA* or *fmhC* were grown at 43°C in BHI to prevent excision of the plasmid. The parental strain was grown under the same conditions without erythromycin.

3. Results

3.1. Identification and characterization of FmhA, FmhB, and FmhC

The FemA and FemB protein sequences were used to search a protein database derived from the genome sequence of the Mc^r clinical isolate of *S. aureus* (ATCC 55748). Three new proteins (Fem homologues: FmhA, FmhB, and FmhC) of similar sizes (416, 421, and 414 aa, respectively) and significant sequence identities were identified (Table 1). Sequence comparisons with the known staphylococcal FemAB-like proteins showed that FmhA and FmhC had stronger homology to each other (59% sequence identity) than to either FemA or FemB (Table 1). Furthermore, FmhA and FmhC were even more similar to Epr [13] and Lif [14], both known to direct serine incorporation into the interpeptide and thus protect the cell wall against digestion by glycylglycine endopeptidases such as lysostaphin or Ale-1. FmhB had the lowest identity to any of these factors. Similar low identities were shown by the non-staphylococcal FemAB-like factors Zif, the zoocin immunity factor [15], and a FemA-like protein reported in *Borrelia burgdorferi* (accession number SPTREMBL O51533), both of unknown function. The 5′ proximal sequence of *fmhB* has very good agreement with Gram-positive consensus promoter sequences [16] and RBS, whereas no such high homologies could be found in front of *fmhA*. *fmhC* (synonym *eprh* [17]) is preceded by *lytN* encoding a putative

Table 1
Amino acid identities of the Fmh factors to other FemAB-like factors

	FemA (Sa)	FemA (Se)	FemB (Sa)	FemB (Se)	Epr	Lif	FmhA	FmhC	FmhB
FemA (Sa)	100								
FemA (Se)	81	100							
FemB (Sa)	40	39	100						
FemB (Se)	40	39	87	100					
Epr	40	41	38	38	100				
Lif	41	43	39	39	70	100			
FmhA	43	41	40	41	62	64	100		
FmhC	37	38	37	37	58	57	59	100	
FmhB	27	26	26	25	23	23	25	21	100

The sequences of FmhA, FmhB and FmhC can be obtained from GenBank under accession numbers AF106849, AF106850, and AF106851. Sa, *S. aureus*; Se, *Staphylococcus epidermidis*. FemA (Se), accession number U23713; FemB (Se), accession number U23714. FmhC is nearly identical (>99.5% identity at the nt level) to the recently published Eprh [17]. *epr*, lysostaphin endopeptidase resistance gene from *Staphylococcus capitis* [13]; Lif, lysostaphin immunity factor from *Staphylococcus simulans* biovar *staphylolyticus* [14].

Table 2
Relative amounts of muropeptides in parent and mutant strains at different growth temperatures

Strain (growth temperature)	Gly_0	Gly_5	Crosslinkage (%)
BB270 (37°C)	1	1	75.3
BB270 (42°C)	5.9	2.6	63.9
fmhA (42°C)	7.5	2.4	66.0
fmhC (42°C)	6.0	2.3	63.9

Gly_0, unsubstituted muropeptide; Gly_5, pentaglycine muropeptide, for structure see [12].

autolysin, and may be cotranscribed with that gene.

3.2. Gene disruption of fmhA and fmhC

Using the *fmhA*, *fmhB* and *fmhC* sequences, internal fragments of the corresponding genes were amplified from the laboratory strain BB270 and sequenced. The amplificates showed less than 1% nucleotide substitutions in the primary sequence of which approximately every fourth nt substitution led to a conservative exchange in the deduced amino acid sequence compared to ATCC 55748. With these fragments, *fmhA* was mapped by Southern blot on the chromosomal *Sma*I-C, *fmhB* on the *Sma*I-H and *fmhC* on the *Sma*I-A fragment of BB270 (data not shown).

These fragments cloned into pOX7 were used for insertional inactivation of the corresponding genes. Disruptants of *fmhA* and *fmhC* were obtained with a frequency of 10^{-3}–10^{-4}. Analysis of their chromosomal DNA by PCR confirmed the integration of the ts plasmids at the expected sites and the absence of an intact wild-type gene of *fmhA* and *fmhC* (not shown). To prevent potential plasmid excision, these strains were maintained and grown at the nonpermissive temperature.

By the same approach no viable disruptants were obtained with the *fmhB* insert, nor did we obtain any integrations using the alternative ts vector pTS1 with a 300 nt long internal *fmhB* insert. Allele replacement by the double crossover approach, which should have substituted part of *fmhB* by an erythromycin resistance cassette, yielded only erythromycin- and chloramphenicol-resistant mutants due to a single crossover resulting in an interrupted and an intact gene copy of *fmhB*. No loss of chloramphenicol resistance, which would indicate a putative double crossover event, was ever observed among over 1000 colonies tested. In a second approach, to protect putative osmotically fragile *fmhB* mutants, the growth medium was substituted in the selection process with hypertonic medium, however, resulting again in no plasmid excision. Addition of 8 μg lysostaphin ml^{-1} also failed to result in double crossovers, although lysostaphin is known to select for *femAB* mutants with shortened pentaglycine interpeptides [2]. Since disruption of *fmhB* proved impossible by these various approaches, and was well below the knockout frequency of this method reported by Brückner [11] that lies in the range of 1% to $>50\%$, whereas *fmhA* and *fmhC* disruptions were functional, we postulate that *fmhB* is essential for *S. aureus*.

3.3. Antibiotic resistance and cell wall composition

The inactivation of *femhA* or *fmhC* had no effects on the resistance levels at 43°C towards vancomycin (MIC 1.5 μg ml^{-1}), teicoplanin (MIC 0.5 μg ml^{-1}), oxacillin (MIC 0.5 μg ml^{-1}), lysostaphin (MIC 0.125 μg ml^{-1}), or methicillin (MIC 1.5 μg ml^{-1}) compared to those of the parental strain BB270 at the same temperature. The MICs of methicillin and of oxacillin of strain BB270 at 37°C were 8 and >256, respectively, as expected. Likewise peptidoglycan composition was identical in the parental and *fmhA* or *fmhC* mutant strains. The elevated growth temperature used to prevent plasmid excision in the mutants led to an increased amount of monomeric muropeptides in the cell wall. Most prominent were the unsubstituted muropeptides without a glycine side chain (Table 2). The increase in these muropeptides was also observed in the parental strain when cultured at 43°C. It caused a slight reduction in overall crosslinking.

4. Discussion

The two *femAB*-like sequences *fmhA* and *fmhC* are of lesser importance and their activity and function are still unknown. They have higher identities to the serine-incorporating Lif and Epr than to the glycine-incorporating FemA and FemB. Interestingly, *fmhC*

is associated with a autolysin-like gene, similar to *lif* and *epr*. Since their inactivation had apparently no effects on growth, cell wall composition or resistance, and since in this *S. aureus* strain no measurable serine substitutions were found in the pentaglycine interpeptide, they may be remnants of FemAB-like proteins, with specificity for amino acids other than glycine, such as serine or alanine which sometimes occur at very low frequencies in the interpeptide of some *S. aureus* strains, but generally more frequently in other staphylococcal species [18]. In contrast, FmhB, the most distantly related of the three new factors, seemed essential for growth, as was also recently observed by Tang et al. [19]. Because of its relatedness to FemA and FemB and its apparent essentiality, FmhB may be the factor FemX predicted to be involved in the addition of the first glycine to the peptidoglycan ε-L-lysine [3]. It may also correspond to the *femAB*-like factor identified in a murine model of bacteremia shown to be required for virulence, as were also *femA* and *femB* [20].

Increased amounts of monomeric muropeptides were observed at elevated temperatures in parent and mutant strains. Although the amount of unsubstituted muropeptides increased six- to sevenfold, it is important to note that this muropeptide is usually a minor component of the staphylococcal cell wall representing only 0.2% of all muropeptides. Thus the resulting reduction in crosslinking comprised only 10%, presumably not enough to account for the drastic drop in methicillin resistance at elevated temperatures. Nevertheless this observation may indicate that the FemX, FemA, and FemB proteins involved in the interpeptide biosynthesis and the PBPs catalyzing the crosslinking may be less active at higher temperatures.

FemAB-like factors may form a new group of proteins involved in non-ribosomal protein biosynthesis. They can be classified into four groups [1]: (i) the Lif- and Epr-containing group that may also integrate serine, to which FmhA and FmhC likely belong; (ii) FemB and (iii) FemA-like factors, which both integrate glycine residues at distinct positions along the interpeptide, and (iv) the more distant FmhB of unknown function.

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